

## Spatial and temporal targeting of gene expression in *Drosophila* by means of a tetracycline-dependent transactivator system

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### SUMMARY

In order to evaluate the efficiency of the tetracycline-regulated gene expression system in *Drosophila*, we have generated transgenic lines expressing a tetracycline-controlled transactivator protein (*tTA*), with specific expression patterns during embryonic and larval development. These lines were used to direct expression of a *tTA*-responsive promoter fused to the coding region of either the  $\beta$ -galactosidase or the homeotic protein Antennapedia (ANTP), under various conditions of tetracycline treatment. We found that expression of  $\beta$ -galactosidase can be efficiently inhibited in embryos and larvae with tetracycline provided in the food, and that a simple removal of the larvae from tetracycline exposure results in the induction of the enzyme in a time- and concentration-dependent manner. Similar treatments can

be used to prevent the lethality associated with the ectopic expression of ANTP in embryos and, subsequently, to control the timing of expression of the homeoprotein ANTP specifically in the antennal imaginal disc.

Our results show that the expression of a gene placed under the control of a tetracycline-responsive promoter can be tightly controlled, both spatially by the regulatory sequences driving the expression of *tTA* and temporally by tetracycline. This provides the basis of a versatile binary system for controlling gene expression in *Drosophila*, with an additional level of regulation as compared to the general method using the yeast transcription factor GAL4.

Key words: Tetracycline, Gene expression, *Drosophila*, Antennapedia

### INTRODUCTION

An essential and general experimental approach to analyse the function of a gene in a whole organism is to examine the phenotypic consequences of its directed expression in certain cells, or at a developmental stage, in which the gene is normally silent. In *Drosophila*, two major systems have been designed to achieve the conditional expression of gene constructs integrated into the genome. In the first, the coding sequence of the gene of interest is placed under the control of promoters inducible according to the culture conditions. The *hsp70* gene promoter is commonly used for that purpose (see Schneuwly et al., 1987 and references therein). High levels of induction can be obtained at well-defined time periods during development upon exposure of the organism to elevated temperatures. This advantage is often limited because ectopic expression occurs in all cells and endogenous genes are repressed during heat shocks. Consequently, side defects including lethality may mask the result of the ectopic expression in the desired cell type(s). It is also frequently necessary to repeat heat shocks over an extended time period to observe the phenotypic consequences, and it may be difficult to distinguish between the primary and secondary effects of the overexpression (e.g. Gibson and Gehring, 1988).

An alternative approach is to engineer a gene construct inducible by a single transcription factor whose activity can be controlled *in vivo*. The ability of the yeast transcription factor GAL4 to activate transcription in *Drosophila* (Fisher et al., 1988) has been exploited to generate a versatile method for targeting gene expression in this organism (Brand and Perrimon, 1993). Any gene of interest can be placed under the control of a promoter containing GAL4 upstream activating sequences (UAS) and integrated stably into the genome of a parental line (responder strain), since it remains silent in the absence of GAL4. Tissue-specific expression of the gene is obtained upon crossing to a second parental line (driver strain) expressing the transcription factor under the control of a suitable promoter. Although a large number of strains expressing GAL4 in a wide variety of patterns can be selected on the basis of the expression of a reporter gene bearing the UAS sequences (Brand and Perrimon, 1993; Yeh et al., 1995; Calleja et al., 1996), only a few of them have been used to direct expression of functional proteins in the post-embryonic stages of development (Brand and Perrimon, 1993; Capdevila and Guerrero, 1994; Hinz et al., 1994; Speicher et al., 1994; Rimmington et al., 1994; Ferver et al., 1995; Halder et al., 1995; Zink and Paro, 1995; Freeman, 1996; Morimura et al., 1996). The limit in this binary system lies in the lack of

temporal control, which remains primarily determined by the regulatory sequences driving GAL4. Because most of the gene-specific enhancers are active at various stages of development in *Drosophila*, GAL4-mediated induction is frequently observed from the embryonic stage onwards, and often results in premature lethality (e.g. Halder et al., 1995). To overcome this difficulty, Flp-mediated recombination has been used to achieve conditional expression of a transgene upon recombination of an FRT cassette separating the coding region from a promoter (Struhl and Basler, 1993). This approach can be used to control the expression of GAL4 (Pignoni and Zipursky, 1997). In any case it requires the combination of various specific constructs and it can be applied only to tissues in which cell division occurs, since it results in the generation of clones of expressing cells.

Extension of the more versatile binary system for post-embryonic studies could be achieved with the use of a regulatory protein modulated in the organism with an innocuous effector. One of the best candidates is the tetracycline-dependent transactivator (*tTA*) comprising the tetracycline repressor of *E. coli* (*tetR*) and the strong transcriptional activation domain of the herpes simplex virus protein VP16 (Gossen and Bujard, 1992). The high affinity and specific binding of *tetR* to the tetracycline operator sequences (*tetO*) can be inhibited by tetracycline (Hillen and Wissmann, 1989) and is thought to result from a conformational change of *tetR* upon association with tetracycline (Hinrichs et al., 1994). In HeLa cells, *tTA* was found to stimulate transcription of a promoter bearing a multimerized *tetO* by several orders of magnitude, and a fast and reversible switch of the *tTA*-dependent promoter was obtained upon addition or removal of tetracycline from the culture medium (Gossen and Bujard, 1992). These features have been extensively exploited in tissue culture where tetracycline levels can be tightly controlled. Tetracycline-regulated expression of reporter genes was also demonstrated in whole plants (Weinmann et al., 1994) and in transgenic mice (Hennighausen et al., 1995; Kistner et al., 1996). In the latter case, the efficiency of tetracycline, administered by slow-release tetracycline pellets or in drinking water, has been mostly evaluated by analysing the expression of sensitive reporter genes, although a few examples of successful expression of proteins have been reported (see Shockett and Schatz, 1996 for a review).

In this study we determined the functional properties of *tTA* in *Drosophila* by expressing this regulatory protein under the control of various promoters. Using a *lacZ* reporter gene placed under the control of a promoter bearing seven copies of *tetO*, we found that expression of  $\beta$ -galactosidase can be tightly controlled in embryos and larval tissues. Furthermore, we used *tTA*-expressing strains and tetracycline treatments to drive tissue-specific ectopic expression of the homeoprotein *Antennapedia* (*Antp*) at different stages of development. Our results demonstrate the usefulness of the *tet* system in *Drosophila*.

## MATERIALS AND METHODS

### DNA constructs

Most of the constructs were assembled by multiple step cloning

according to standard methods (Sambrook et al., 1989). Further details and maps are available upon request.

### *tTA* driver constructs

#### *hsp70-tTA*

The *tTA* coding region was isolated as a 1.1 kb *EcoRI*-*Bam*HI fragment from pUHD 15-1 (Gossen and Bujard, 1992) and cloned into CaSpeR-hs (Thummel and Pirrotta, 1992).

#### RHT (*rosy*, *hsp70* promoter, *tTA*)

This P element vector derives from the enhancer-test vector HZ50PL (Hiromi et al., 1985). The *tTA* coding region is flanked by the minimal promoter and the poly(A) sequences of *hsp70* (Fig. 1A).

#### *ey-tTA* and *HoxA7-tTA*

The *eyeless* (*ey*) gene enhancer (a 3.5 kb *Kpn*I fragment from *ey* Eco 3.6) (B. Hanck, T. Eggert, W. J. Gehring and U. Walldorf, unpublished) and a 630 bp fragment of the intron of the *HoxA7* gene from pB6 (Haerry and Gehring, 1996) were cloned in RHT to give *ey-tTA* and *HoxA7-tTA*, respectively.

### Tetracycline-responder constructs

#### *tetO-lacZ*

The heptameric repeat of the tet operator was isolated as a 310 bp *EcoRI*-*Kpn*I fragment from pUHC 13-3 (Gossen and Bujard, 1992) and cloned upstream of the P-*lacZ* fusion of the enhancer-test vector CPLZ (Wharton and Crews, 1993). CPLZ contains the P-element transposase promoter (up to -42 from the cap site) and the N-terminal transposase sequence fused in-frame with *lacZ* and the polyadenylation signal of *hsp70*.

#### WTP (white-*tetO*-P promoter)

This P-element vector was constructed to express any gene under the control of a tetracycline-responsive promoter. It contains the vector backbone of CPLZ, the heptameric repeat of the tet operator, the P-element promoter and leader sequences from Carnegie 4 (Rubin and Spradling, 1983) and the polyadenylation signal of SV40.

#### *tetO-Antp* and *tetO-Antp $\Delta$ HD*

The cDNAs encoding a full-length ANTP protein or a variant with a deletion of the homeodomain were isolated as *Nor*I fragments from pHSSAA and pNHT-A11, respectively (Gibson et al., 1990) and cloned into the corresponding site of WTP.

### Germline transformation and *Drosophila* strains

P-element mediated transformation of *ry<sup>506</sup>* or *y ac w<sup>1118</sup>* recipient strains was carried out essentially as described (Spradling, 1986). A description of the markers and balancer chromosomes indicated in Figs 3 and 4 can be found in Lindsley and Zimm (1996). A405.1 M2 and rK781 have been described (Wagner-Bernholz et al., 1991; Flister, 1991).

### Tetracycline media and treatments

A tetracycline-containing medium suitable for larval feeding and maintenance of adults was obtained by mixing 100 ml of tetracycline solution (tetracycline hydrochloride (Sigma) diluted with sterile water at the required concentration), 25 g of Instant *Drosophila* Food (Carolina Biological Supply) and 1 g of dry yeast.

Tetracycline was provided to adult females by placing 50-70 virgins in a glass vial containing a 2.5 cm filter (Whatman grade 3 MM) soaked with 500  $\mu$ l of a 4% sucrose solution with tetracycline at the appropriate dilution. Females were fed for 3-4 days, following a daily cycle of 16 hours on tetracycline-containing filters and 4 hours on standard food supplemented with a drop of yeast paste. Males of the relevant genotype were placed with females on tetracycline and allowed to mate overnight before the beginning of egg collections. For

experiments requiring larval feeding, batches of 100-200 eggs harvested from grape juice plates were placed on pieces of nylon mesh, and allowed to develop at 25°C on tetracycline-containing food. When necessary, larvae were separated from their food by floating in 30% glycerol, collected with forceps, washed with PBS and transferred on standard food in groups of 50 to 100.

### Phenotypic analyses

Embryos and larval tissues were fixed and stained for  $\beta$ -galactosidase as described (Bellen et al., 1989). Adult heads were separated from the body of narcotised flies and holes were made into the cuticle to facilitate penetration of the fixative. For antibody staining of embryos and examination of cuticular phenotypes, standard procedures were applied (Ashburner, 1989).

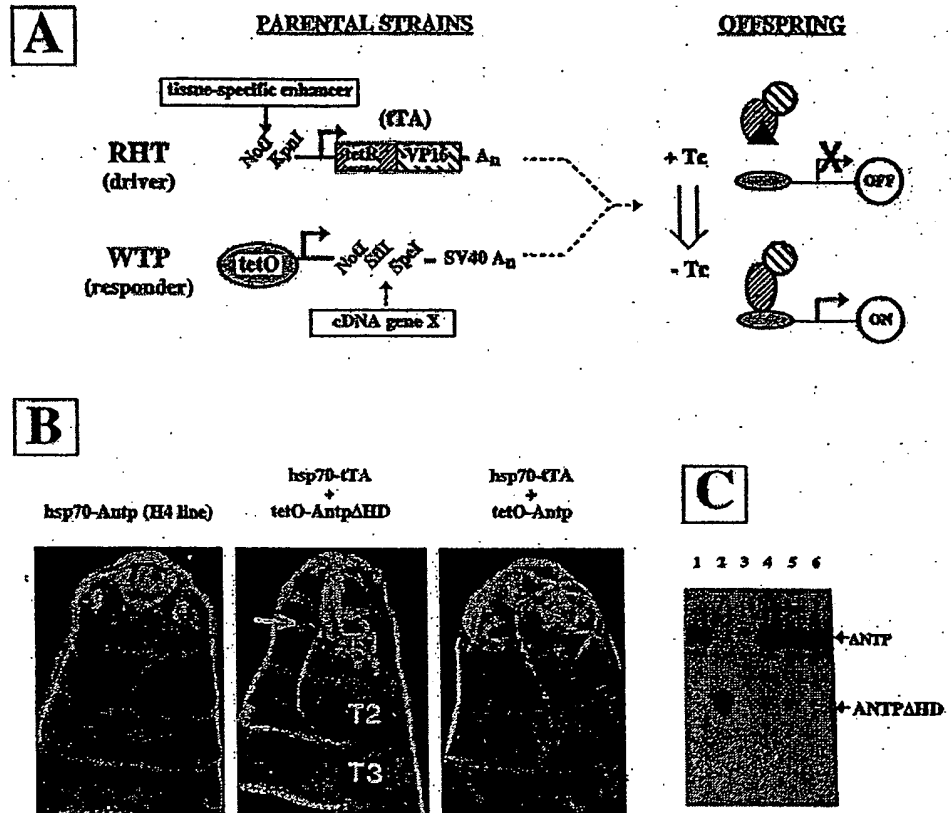
## RESULTS

We have designed a general system to express *tTA* under the control of regulatory sequences (RHT driver construct), in order to direct the expression of a gene of interest under the regulation of a tetracycline-responsive promoter (WTP responder construct). The gene constructs can be stably propagated into the genome of separate strains, and *tTA*-dependent gene induction is obtained in the F<sub>1</sub> offspring of the cross where it can be controlled by tetracycline (Fig. 1A).

### *tTA* is a potent transactivator in *Drosophila*

In order to analyse the transactivation potential of *tTA*, we have used an indirect heat shock assay to drive ubiquitous expression of *Anp* in embryos. Heat shock assays were performed on embryos carrying *tTA* under the control of the *hsp70* gene promoter (*hsp70-tTA*) and a WTP derivative carrying a full-length *Anp* cDNA (*tetO-Anp*). Independent transformants were found to give an identical embryonic phenotype to the H4 line that carries a direct *hsp70-Anp* construct (Fig. 1B; see also Gibson and Gehring, 1988 for a complete description of the H4 line). In contrast, heat-shocked embryos carrying a WTP derivative with a deletion of the homeodomain (*tetO-Anp $\Delta$ HHD*) or the empty WTP vector (*tetO-*) showed a wild-type cuticle and developed to the adult stage (Fig. 1B and data not shown). A western blot of embryonic extracts prepared from

heat-shocked embryos and probed with an ANTP-specific monoclonal antibody (Condie et al., 1991) reveals similar levels of ANTP expression (Fig. 1C). In addition, transformation of the adult antenna into a mesothoracic leg can be obtained when heat shocks are applied to third instar larvae (Gibson and Gehring, 1988; D. Resendez-Perez, B. Bello and W. J. Gehring, unpublished). Taken together, these results show that *tTA* can activate transcription of a promoter that contains *tetO* sequences without any toxic effect of this regulatory protein in *Drosophila*.



**Fig. 1.** Transgenic constructs and the transactivation potential of *tTA* in *Drosophila*. (A) Schematic representation of the tetracycline-inducible system in *Drosophila*. The transformation vectors RHT and WTP are, respectively, designed to express *tTA* under the control of enhancer-like elements and any cDNA under the regulation of a *tTA*-responsive promoter that contains *tetO* sequences. Derived constructs are integrated and propagated in two separate parental strains. In absence of tetracycline (-Tc), the binding of *tTA* results in the activation of gene X with the spatial and temporal specificities determined by the driver construct (on state). Raising the offspring on a tetracycline-containing medium is used to prevent the binding of *tTA* in order to keep the responder construct silent up to a certain point of time (off state). Stopping tetracycline exposure allows time-specific induction of gene X upon withdrawal of the antibiotic. (B) Cuticular preparations of embryos heat shocked for 30 minutes at 6.5 hours of embryogenesis. All embryos carry a single copy of the constructs indicated at the top. Note the similar defect in head evolution in *hsp70-Anp* and *hsp70-tTA; tetO-Anp*, and the T1 to T2 transformation when compared to the wild-type cuticle of *hsp70-tTA; tetO-Anp $\Delta$ HHD* embryos. The identical segmental transformation is revealed by the disappearance of the characteristic denticle belt of the first thoracic segment (white arrow), indicating a transformation of T1 towards T2. The head segments are also transformed towards T2. T1, T2, T3: first, second and third thoracic segments respectively. (C) Detection of the ANTP proteins by western blot analysis in crude extracts of embryos heat shocked for 2 hours with a 4 hour recovery. Lane 1, *hsp70-Anp* embryos (H4 line); lanes 2-6, embryos carrying *hsp70-tTA* and either *tetO-Anp $\Delta$ HHD* (lane 2) or the empty WTP vector (lane 3), or independent insertions of *tetO-Anp* (lanes 4-6).

### Tetracycline-controlled expression of *lacZ* during larval development

In order to express *tTA* during larval development, we inserted the eye-specific enhancer of the *ey* gene (Quiring et al., 1994) into the vector RHT and generated *ey-tTA* transformants. The expression of *tTA* was detected specifically in the eye imaginal disc by means of a *lacZ* reporter gene placed under the control of a promoter bearing *tetO* sequences (Fig. 2). Detection of  $\beta$ -galactosidase activity in the eye disc of the larvae reveals two essential features. First, enzymatic activity is detectable within less than 15 minutes of incubation with X-gal revealing a high level of expression of *tTA*. Second, this activity is detected in the eye disc over an extended period of development with a dynamic pattern (Fig. 2A, top row). In the early third instar, expression is detected uniformly in the eye part of the eye-antennal disc (left panel), which corresponds to the undifferentiated cells of the eye epithelium (Ready et al., 1976). The same pattern was observed in second instar larvae (not shown). Eye discs stained at different times during the third instar show that  $\beta$ -galactosidase activity gradually fades in the anterior region of the disc as the morphogenetic furrow moves anteriorly.

To determine whether tetracycline incorporated in the larval food could inactivate *tTA*, we stained early third instar larvae raised on media containing increasing concentrations of the antibiotic (Fig. 2A, time 0). No activity could be detected in the eye-disc of larvae exposed to as little as 0.1  $\mu\text{g/ml}$  even after prolonged staining (24 hours) in X-gal solution (Fig. 2A, left column). Lower concentrations failed to inactivate *lacZ* expression (not shown). The results indicate an efficient uptake of tetracycline from the food and its diffusion to the imaginal discs, leading to a complete inactivation of *tTA* in a concentration-dependent manner. The same dose-response was obtained for larvae exposed to tetracycline for approximately 2 more days, suggesting that the concentration of tetracycline in the larval haemolymph does not change dramatically during the feeding period (not shown). In contrast, stopping the larval exposure to tetracycline was expected to decrease the level in the haemolymph and to restore the ability of *tTA* to bind and transactivate the *lacZ* reporter gene. To test this hypothesis, early third instar larvae were transferred to standard food, fixed and stained every 6 hours for  $\beta$ -gal activity (Fig. 2A). Enzymatic activity was detected 6 hours after the removal of the larvae from the medium containing 0.1  $\mu\text{g/ml}$  tetracycline

(Fig. 2A, second row from the top) or in a range of 18 to 24 hours when the larvae were exposed to 1  $\mu\text{g/ml}$  or 10  $\mu\text{g/ml}$  tetracycline, respectively (Fig. 2A, third and fourth row from the top). After induction, 12-24 hours were necessary to obtain

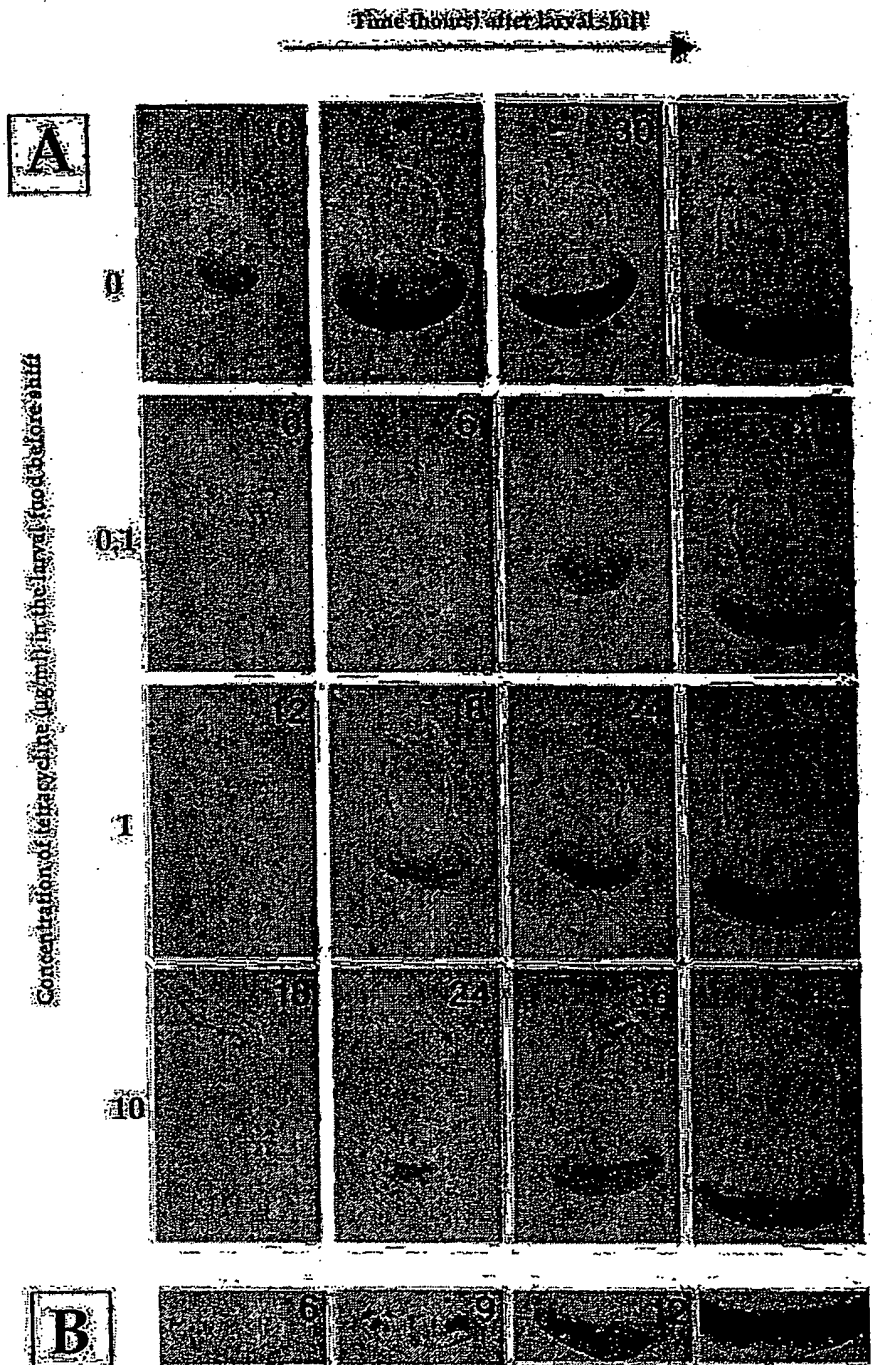


Fig. 2. Tetracycline controlled expression of *lacZ* in the eye imaginal disc driven by the *ey-tTA* strain. (A) Larvae of the genotype *ey-tTA/+; tetO-lacZ/+* were raised in the presence of increasing concentration of tetracycline, shifted to standard food at the early third instar and dissected at different times after shifting, indicated in hours at the top right of every figure. (B) Temporal profile of *lacZ* induction from larvae removed from 0.1  $\mu\text{g/ml}$  tetracycline during the late third instar. Note the increasing number of cells expressing *lacZ*.

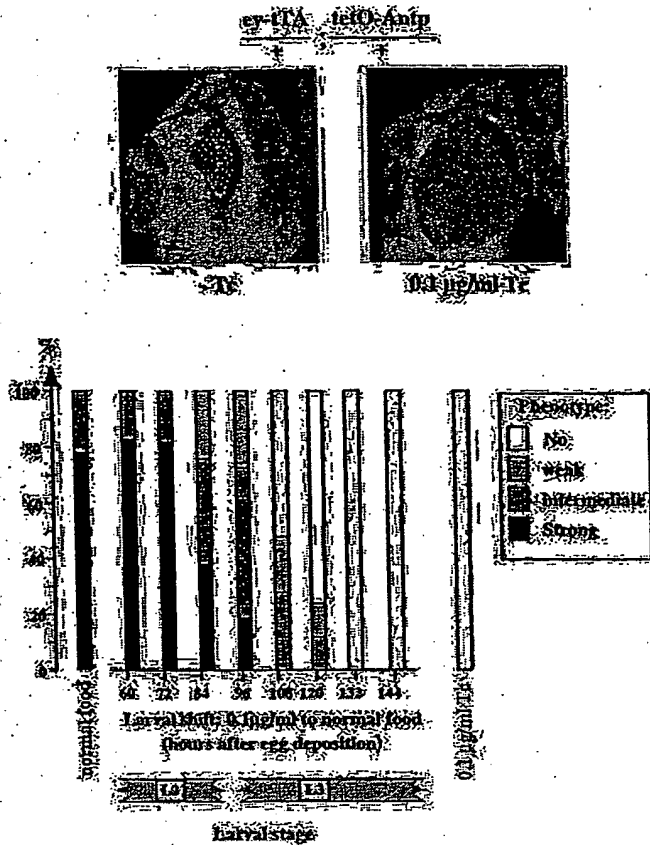


Fig. 3. Induction of *Antennapedia* in the eye imaginal disc by means of tetracycline. (Top) Eyes of adult flies raised in absence (-Tc) or in the presence of 0.1  $\mu$ g/ml tetracycline (Tc) in the larval food. (Bottom) Semi-quantification of the eye phenotype in adults derived from larvae raised with 0.1  $\mu$ g/ml tetracycline and shifted to standard food at 12 hour intervals. At least 50 adults were scored at every time point. Larval stages were identified on the basis of the morphology of the tracheal system and the anterior spiracles.

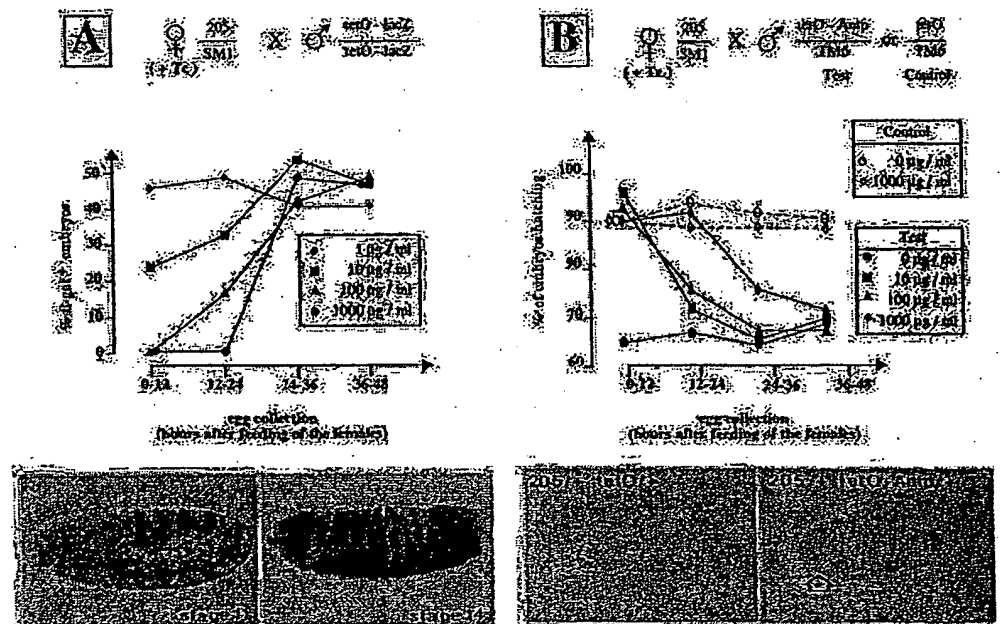
followed by a rapid induction of the *tetO-lacZ* transgene under the control of *tTA*. The concentration- and time-dependent expression of *lacZ* is likely to reflect the need to lower the concentration of tetracycline in the disc cells below a certain threshold level, which allows *tTA* to bind the tet operator and to stimulate transcription of the promoter. A careful examination of the staining patterns also suggested that the *tetO-lacZ* transgene was not turned on in every cell at the same time after withdrawal of tetracycline (Fig. 2A). To confirm this observation, we performed larval shifts during the second half of the third instar, when expression of *tTA* is uniform in the posterior part of the eye disc. Upon removal of the larvae from 0.1  $\mu$ g/ml tetracycline, induction of *lacZ* can clearly be observed in a gradually increasing number of cells (Fig. 2B). Similar observations were obtained with different *tTA*-expressing strains, suggesting variations in the kinetics of the clearance of the antibiotic and/or the transcriptional activation.

#### Tetracycline-controlled expression of *Antennapedia* targeted to the eye disc

To determine the relevance of the data obtained with the *lacZ* reporter gene we used the same driver strain to direct expression of *Antp* under various conditions of tetracycline treatment. In the absence of tetracycline, adults obtained from

a comparable staining intensity in imaginal discs isolated from larvae exposed to tetracycline and control larvae. These data show that the withdrawal of the larvae from tetracycline is

Fig. 4. Inhibition of *tTA*-dependent gene expression in embryos by maternal transmission of tetracycline. (A) Repression of the *lacZ* reporter driven by the 205 strain, shown at the bottom by immunodetection of  $\beta$ -galactosidase. Embryos collected over successive 12 hour periods following the feeding of 205/SM1 females with tetracycline, were stained for  $\beta$ -gal activity. At least 100 embryos older than the germ band extended stage (stage 11) were scored at every time point. 50% of transheterozygotes were expected in the offspring of the cross indicated at the top. (B) Tetracycline-dependent rescue of embryonic lethality by repression of the *tetO-Antp* transgene. Embryos collected over 12 hour periods after feeding of their mothers with tetracycline were allowed to develop at 25°C and the percentage of hatching larvae determined. At least 200 embryos were scored at every time point. 25% of transheterozygotes are expected from the cross indicated at the top. *tetO* refers to an insertion of the empty vector WTP on the third chromosome, used as a control. Bottom: cuticular phenotype of embryos of the indicated genotype. Note the failure of head involution marked by the open arrow in embryos expressing *Antp* under the control of the 205 strain.



a cross between the parental *ey-tTA* and *tetO-Antp* strains showed reduced and irregular compound eyes (Fig. 3, -Tc). Since *Antp* is normally not expressed in the eye disc (Wirz et al., 1986), its expression in this tissue would interfere by unknown mechanisms with the normal development of the eye. No other morphological defects could be detected in adults, in agreement with the eye-specific expression of *tTA* detected with the *lacZ* reporter. To ascertain that the eye phenotype resulted from the ectopic expression of *Antp* in the eye disc, we raised larvae on a medium containing tetracycline at various concentrations. The eyes were restored to a wild-type appearance with 0.1 µg/ml tetracycline (Fig. 3, top) but not with 0.01 µg/ml tetracycline, in good agreement with the dose-dependent repression of the *lacZ* reporter (Fig. 2A).

As indicated with the *lacZ* reporter, the directed expression of *Antp* by the *ey-tTA* strain should occur continuously throughout larval development and shift rapidly during the third instar when the cells undergo differentiation. We used tetracycline to control the timing of *Antp* overexpression in order to determine the functional significance of this dynamic pattern of expression, with respect to the alteration of the eye development. Newly hatched larvae were first fed with 0.1 µg/ml to inhibit *tTA* activity and then transferred to a standard medium every 12 hours to induce *Antp*. Adults were scored for eye defects and classified according to an arbitrary scale of strong, intermediate, weak or no detectable eye phenotype (Fig. 3, bottom). All the adults derived from larvae exposed to tetracycline up to the second instar showed strong eye defects in a range indistinguishable from their siblings raised in absence of tetracycline. In contrast, subsequent shifts allowing induction of *Antp* from the early third instar onward led rapidly to a complete rescue of the eye morphology. These results confirm the efficiency of the tetracycline control with a functional homeoprotein and suggest that the alteration in the normal eye development is mostly dependent on the ectopic expression of *Antp* in the undifferentiated cells of the eye epithelium.

#### Repression of *tTA*-dependent gene expression in embryos by maternal transmission of tetracycline

The embryonic development of *Drosophila* is not easily amenable to antibiotic treatment since the egg is protected by an impermeable set of eggshells but it is relatively fast (22–24 hours at 25°C) and maternal components are transmitted to the oocyte by the nurse cells and the follicle cells in the female ovaries. The influence of tetracycline given to the parental females was first tested on the strong *lacZ* expression driven by the *tTA* construct of the line 205 (Fig. 4A). This driver line 205 was isolated among twelve independent transformants of the *HoxA7-tTA* construct (see Materials and Methods) because of its unique expression pattern observed in the antennal disc (Fig. 5), the leg discs, the central nervous system, the epidermis and various internal tissues (not shown). Since the other lines showed a reproducible pattern in the eye disc and the larval brain due to the *HoxA7* enhancer (not shown), the line 205 is likely to reflect a modified expression of the transgene under the influence of genomic regulatory sequences flanking the integration site (Wilson et al., 1990). When assayed in embryos with the *lacZ* reporter gene, expression of *tTA* in the 205 strain starts at the end of germ band extension, about 5 hours after egg laying (AEL), and is detected mostly in the trunk region

with a segmentally repeated pattern (Fig. 4A, bottom). This pattern changes rapidly, so that at the end of germ band retraction (approximately 10 hours AEL), strong expression is detected all over the ectoderm. The staining appears patchy in the cephalic segments and is not uniformly distributed in the thoracic and abdominal segments (Fig. 4A, bottom). After treatment of the females with tetracycline (see Materials and Methods), repression of the *lacZ* reporter is mostly effective in the eggs collected immediately after the end of exposure to the antibiotic and is dose-dependent (Fig. 4A, top). Repression was obtained in 100% of the eggs collected within 12 hours after the treatment of the females with 100 µg/ml or more of the antibiotic, and in a large fraction of them with 10 µg/ml. The gradual loss of repression observed in the eggs collected later is likely to reflect a decrease of the maternal pool of tetracycline accompanying the continuous production of eggs.

The same procedure of tetracycline treatments was tested for its effect on the survival rate of embryos carrying the driver construct 205 and either a *tetO-Antp*, or an empty responder construct (*tetO-*), as control (Fig. 4B). Examination of the embryonic cuticles revealed major defects in the formation of the head (Fig. 4B, bottom), a phenotype reminiscent of heat-shocked embryos in which *Antp* was ubiquitously expressed (Fig. 1B), although no homeotic segmental transformations were observed. This might reflect a different level of induction of *Antp* as compared to the use of a heat shock promoter but it is more likely to be due to a difference in the timing and the spatial expression of the homeoprotein, since transformations obtained by heat shocks are optimal when induced at 5–7 hours of development (Fig. 1B, see also Gibson and Gehring, 1988), at a stage when the 205 driver is mostly active in the trunk and is not ubiquitously expressed (Fig. 4A, bottom). Nevertheless, line 205 allowed us to test the effect of tetracycline on the survival rate of the embryos and, as shown in Fig. 4B, the embryonic lethality could be overcome in a dose-dependent manner by providing tetracycline to the females. The embryonic rescue was in good agreement with the tetracycline-mediated repression of the *tetO-lacZ* transgene (Fig. 4). These two independent assays clearly demonstrate the possibility to inactivate *tTA* in embryos in order to keep a promoter silent during this stage of development.

#### Targeted mis-expression of *Antp* in larvae following embryonic rescue

The efficient inactivation of *tTA* in embryos prompted us to analyse the fate of tetracycline-rescued embryos in more detail. As expected, embryos did not develop to the adult stage in the absence of tetracycline in the larval food, whereas addition of tetracycline led to the recovery of viable adults in a concentration-dependent manner (the quantitative data are available upon request). In addition, larvae raised under optimal conditions were shifted to standard food at various times to allow induction of *Antp*. Shifts performed during the late third instar led to the recovery of pharates or adults, whereas larvae shifted earlier essentially failed to undergo metamorphosis. Examination of the adults revealed very specific morphological modifications of the antennae and the head vertex (Fig. 5K–M), in the area expressing the *lacZ* reporter under the regulation of the 205 driver line (Fig. 5E–G). In contrast, transheterozygotes raised continuously with 10 µg/ml tetracycline showed wild-type structures (Fig. 5H–J),



demonstrating the highly specific alterations in the development of adult flies following the mis-directed expression of *Antp* by the 205 line. As revealed with the *lacZ* reporter, expression of *tTA* follows a dynamic spatial pattern in the primordia of the antenna from the mid-third instar onwards (Fig. 5A-D) and in the presumptive area of the ocelli from the larval/pupal transition onward (Fig. 5C,D). Moreover, the lag in induction imposed by the removal of the larvae from tetracycline exposure suggests that the alterations of adult structures mostly result from the ectopic expression of *Antp* during the pupal stage. No defects were found in the legs or the palps where the 205 driver is also strongly expressed (not shown), in agreement with previous observations showing that only the derivatives of the eye-antennal disc respond to the ubiquitous expression of *Antp* induced by heat shock (Gibson and Gehring, 1988). These latter studies showed that repeated pulses of heat-shock expression are required during the third larval instar to achieve complete antenna to leg transformations. Our results confirm previous observations showing that the late larval induction of *Antp* does not induce fully differentiated morphological markers of the leg (Scanga et al., 1995; Larsen et al., 1996). Although we observe different arrangements of bristles on the antenna, none of them showed the bracts characteristic for leg bristles.

#### Directed expression of *Antp* in the antennal disc by *tTA* activates *rK781*

Since the observation of adult phenotype required late larval shifts, we asked whether the consequences of *tTA*-dependent expression of *Antp* could be directly assayed in the imaginal discs. As a marker, we used the enhancer detector line *rK781*, which was isolated in a screen for *Antp*-regulated genes on the basis of their response to the overexpression of the ANTP homeoprotein in the eye-antennal disc (Wagner-Bernholz et al., 1991). We combined driver, responder and test constructs in larvae, exposed them to tetracycline treatment, and assayed  $\beta$ -galactosidase expression in wandering third instar larvae. When raised continuously with 10  $\mu$ g/ml tetracycline, the normal pattern of *rK781* expression was detected in all the discs (not shown) and in a few cells of the antennal disc (Fig. 6, left), as previously described (Wagner-Bernholz et al., 1991; Flister, 1991). When dissected from larvae that were removed from tetracycline exposure, *lacZ* expression could be reproducibly detected in the form of a crescent at the border between the arista and the third antennal segment (Fig. 6, middle). This area corresponds to the most proximal part of the wedge-shaped sector expressing *tTA*, as visualised with the *lacZ* reporter (Fig. 6, right) and is also the first to express *tTA* during third instar (Fig. 5B). These results show that derepression of *Antp* by removal of tetracycline can be demonstrated by the activation of a downstream target gene in the antennal disc. These findings indicate that the tetracycline-dependent expression system efficiently repressed *Antp* in embryos and allows subsequent derepression in imaginal discs.

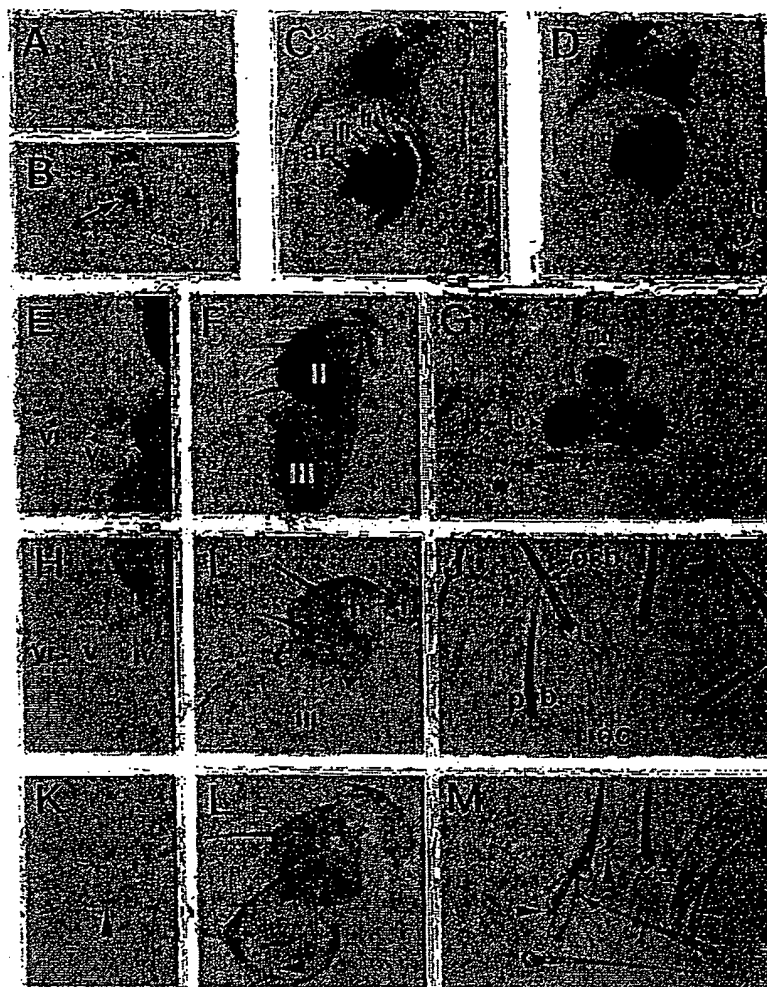
#### DISCUSSION

In this study, we report a detailed evaluation of the different properties of the tetracycline-dependent gene expression system in *Drosophila*. Since its description in transformed HeLa cells (Gossen and Bujard, 1992) this regulatory system

has been extensively used in cell culture. In higher eucaryotes including plants and mouse, tetracycline-controlled activity of *tTA* has been mostly evaluated on reporter genes (see Shockett and Schatz, 1996 for a review), although a few examples of successful expression of proteins have been reported (Efrat et al., 1995; Ewald et al., 1996; Mayford et al., 1996; Shockett et al., 1995; St-Onge et al., 1996). By using *Drosophila* lines expressing high levels of *tTA*, we show that the strong induction of the *lacZ* reporter can be efficiently prevented by tetracycline in both embryos and imaginal discs. We have evaluated the dose-response and defined easy and reliable protocols of tetracycline treatment to control the repression of the *lacZ* reporter gene. Furthermore, we also show that this system is fully functional to control the spatial and temporal expression of the ANTP homeoprotein. The lines *ey-tTA* and 205 described in this study show the highest levels of *tTA* among the lines generated to date in the laboratory and we have reproducibly obtained repression of gene activity in embryos by feeding their mothers with tetracycline in a range from 1 to 1000  $\mu$ g/ml tetracycline, and in larvae, with as little as 0.1  $\mu$ g/ml tetracycline. The use of tetracycline is especially appropriate to keep the inducible gene promoter silent during embryogenesis in order to direct its expression during larval development. Tetracycline concentrations ranging from 0.1 to 10  $\mu$ g/ml ensure reactivation of the tetracycline-responsive promoter within 24 hours after transfer of the larvae to normal medium. It is important to point out that the amount of tetracycline required to inactivate *tTA* is both low and non-toxic. This is essential to keep the promoter inactive up to a desired stage and to ensure its fast activation upon removal from tetracycline exposure. We have found that the addition of tetracycline to the larval food does not give any toxic effect in a range of 0 to 100  $\mu$ g/ml, although the development is slowed down at concentrations above 1  $\mu$ g/ml. As shown with the *ey-tTA* strain, tetracycline can be used to control the timing of induction at distinct phases of development in order to define a phenocritical period. Temporal control of gene expression should also be effective during pupal development as a function of the concentration of tetracycline provided to the larvae before pupation. They can be well synchronised during this developmental period and go through a number of well-characterised stages (Ashburner, 1989). In combination with the use of the *lacZ* reporter, these features should help in determining the time course of induction of any gene driven by a *tTA*-expressing line of interest.

Our attempts to use the reverse tetracycline-controlled transactivator (*rtTA*, Gossen et al., 1995) have been unsuccessful in *Drosophila*. This transactivator is based on a mutagenized version of *tTA*, which binds the *tetO* sequences only in the presence of specific tetracycline derivatives. It corresponds to a 4-amino-acid exchange in *tetR*, which is thought to alter the conformation of the repressor and allows its binding to DNA upon association with certain tetracycline compounds (Gossen et al., 1995). Since it was originally isolated in a genetic screen in bacteria and tested successfully in mammalian cells (Gossen et al., 1995) and in transgenic mice (Kistner et al., 1996), *rtTA* might need a temperature close to 37°C to be stable. In contrast, *tTA* shows a potent activity in *Drosophila* and its negative regulation by tetracycline is not a major difficulty, as described above. Furthermore, both the repression and the kinetics of gene induction might be

**Fig. 5.** Adult phenotype resulting from the directed expression of *Antennapedia* by the 205 strain. (A-D) Expression pattern of the 205 strain in the eye-antennal disc visualized by X-gal detection of  $\beta$ -galactosidase activity encoded by *tetO-lacZ* reporter. Discs are oriented with anterior up and dorsal left. (A) Early third instar. (B) Mid third instar; expression starts in the centralmost region of the antennal disc (arrow) and the presumptive palp region (arrowhead). (C) Late third instar larvae: expression has expanded in a wedge-shaped sector overlapping the most proximal region of the arista (ar) and the three major antennal segments (roman numerals). Expression in the lateral ocellus (lo) that is not detectable in active wandering larvae is also indicated. (D) White prepupae:  $\beta$ -gal activity is detectable in the medium ocellus (mo). (E-G) Expression of the *lacZ* reporter in the respective adult structures: the most proximal segments of the arista (E), the three antennal segments (F) and the ocelli (G). (H-J) Normal phenotype of 205/+; *tetO-Antp*+ adults raised continuously with 10  $\mu$ g/ml tetracycline. Occipital, post vertical and interocellar bristles are indicated by ocb, pvb and ioc, respectively. (K-M) Altered phenotype of 205/+; *tetO-Antp*+ adults derived from larvae shifted from 10  $\mu$ g/ml tetracycline to standard food during the late third instar. Note the thickening of the proximal segments of the arista (K, arrowhead), a bunch of new bristles on the third antennal segment and a modification in the number, the localization and the shape of the characteristic bristles of the second antennal segment (L, arrowheads). We could not determine the origin of these bristles on the basis of their morphology. Although different from the usual antennal bristles, they are not of a leg type because of the lack of the characteristic bract. The other main feature is a bunch of thick and long bristles of unknown origin close to the ocelli (M, arrowheads).



increased further with one of the numerous tetracycline derivatives available. Some of them have been shown to be more potent effectors on *tTA* than tetracycline itself (Gossen and Bujard, 1993; Chrast-Balz and Hooft van Huijsduijnen, 1996).

### Binary systems for controlling gene expression

The interest in using a binary system that combines an effector molecule for controlling activity of a responder promoter has been largely demonstrated with the GAL4/UAS system (Brand and Perrimon, 1993). Our method makes use of a similar experimental strategy, which allows the stable integration of any kind of construct in a parental fly strain in the absence of the transactivator. Tissue-specific activation of the gene construct is achieved in the offspring of a cross with a driver line chosen for its pattern of expression of the transactivator. The *tet* system provides a more versatile tool, owing to the possibility of controlling the timing of gene expression during development. In addition, the use of the vectors RHT and WTP facilitates the generation of driver strains expressing *tTA* under the control of previously isolated tissue-specific enhancers, and responder strains carrying any gene of interest under the regulation of the *tetO*-containing promoter. We also plan to generate a collection of *tTA*-expressing strains following the



**Fig. 6.** Tetracycline-controlled expression of *Antennapedia* by the 205 strain activates *rK781*. Shown are  $\beta$ -galactosidase-stained antennal discs isolated from larvae raised continuously on 10  $\mu$ g/ml tetracycline (+Tc) or shifted to standard food 48-72 hours before dissection (-Tc). The arrow points to the activation of *rK781* in the centralmost region of the wedge-type sector of *tTA* expression, visualized on the right by the *tetO-lacZ* reporter. Eggs were collected over 12 hours from tetracycline-treated females of the genotype Cy[A405. M2]/205; *rK781/rK781* mated with +/+; *tetO-Antp/TM6,Tb* males. Larvae of the genotype 205/+; *tetO-Antp/rK781* were identified by their *Tb*<sup>+</sup> phenotype and the absence of the staining pattern due to the Cy[A405. M2] chromosome.



random integration into the genome of an enhancer detector construct with *tTA* as a reporter gene. The availability of strains expressing *tTA* in a wide variety of patterns will ensure a large number of applications for the *tet* system.

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